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The spread of cell death from impact damaged cartilage: lack of evidence for the role of nitric oxide and caspases[1]

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Summary

Over 21 days in culture, cell death spreads, both radially and transversely, from loaded to surrounding cartilage. This spread was prevented by physical separation and separate culture post-impact.

Objective: One aim was to determine if nitric oxide (NO) is the intercellular signal mediating cell death. Another aim was to clarify the nature of the cell death, whether caspase mediated apoptosis or necrosis.

Design: Cyclic impacts were applied to the central 2 mm core of 4 mm canine articular cartilage discs. Post-impact culturing was for 21 days in the presence or absence of the iNOS inhibitor, L-NAME, or the broad-spectrum caspase inhibitor, Z-VAD FMK. Cell death was quantified using the TUNEL assay. Culture media were collected every 2 days for measurements of glycosaminoglycan (GAG) and NO release.

Results: Cell death spread from the loaded core into the surrounding ring over 21 days in culture. Although L-NAME significantly reduced nitrite release into the culture media of both loaded and control cartilage, the spread of cell death was not prevented. Neither was the spread of cell death prevented by Z-VAD FMK.

Conclusions: These data indicate that NO is not acting as an intercellular signalling factor in this *in vitro* system and that the cell death post-impact is not caspase mediated.

Key words: Articular cartilage, Cell death, Mechanical damage, Nitric oxide.

Introduction

Although the aetiopathogenesis of osteoarthritis (OA) is unknown, there is strong evidence for an increase in the levels of chondrocyte death in osteoarthritic cartilage both in humans[1] and in our canine hip dysplasia model[2]. This occurs alongside further damage to the cartilage matrix, including a decrease in the proteoglycan content[3], an increase in the water content[3] and an increase in the amount of collagen degradation[4]. *In vitro* studies have reproduced these characteristic osteoarthritic changes using models of mechanical load. Mechanical load is a known risk factor for the development of OA and several studies have shown increased cell death post-load[5–8].

Previous work from our laboratory has shown that, over time, cell death spreads from the loaded to the surrounding, unloaded area of cartilage. When the central 2 mm of a 4 mm disc is loaded, initially dead cells were located in the region directly below the indenter. In loaded cartilage explants cultured for 21 days, cell death did not remain localised to the directly impacted region but spread both radially and transversely into the surrounding ring cartilage. Furthermore, this spread could be prevented by physical separation, and separate culture, of the loaded and surrounding cartilage[9]. These data suggest that a soluble factor is functioning as a messenger of cell death following mechanical loading. In this study, we examined nitric oxide (NO) as a potential intercellular signalling molecule. NO is an inorganic free radical, which can act as a cell signalling molecule and which has been shown to inhibit proteoglycan synthesis[10] and increase matrix metalloproteinase activity[11] in cartilage. There are increasing numbers of reports which suggest that NO can cause apoptosis both in cartilage[12] and in isolated chondrocytes[13]. NO itself diffuses easily into and out of cells but has a very short half-life (5–10 s). Changes in NO must be assessed as changes in nitrate plus nitrite concentrations. Also, *S*-nitrosoproteins, formed by NO binding to thiol-containing proteins, permit stable storage of NO within a tissue[14].

Another important question regarding the spread of cell death is the process by which it occurs; that is, is it apoptosis or necrosis? Chen *et al.*[6] showed that following load the initial cell death occurring within 4 h is predominantly necrosis with the percentage of apoptotic cells increasing with time post-load. Cartilage has no phagocytic cells to remove the remnants of dead cells and the accumulation of apoptotic bodies may be important in the pathogenesis of OA[15]. Characteristic morphological changes in apoptosis are brought about by a family of cysteine proteases, caspases, which cleave following aspartic acid residues.

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In this current paper, we hypothesise that NO is the messenger propagating the wave of cell death in cartilage and that this spread of cell death is apoptotic. To test these hypotheses, explants of canine articular cartilage were loaded and subsequently cultured in media supplemented with either the iNOS inhibitor, L-NAME or the broad-spectrum caspase inhibitor, Z-VAD FMK. Twenty-one days post-load, explants were frozen and cell death was quantified using the TUNEL assay.

Methods

CARTILAGE EXPLANTS

Articular cartilage explants were obtained, as 4 mm discs, from canine shoulder joints, under sterile techniques, using a 4 mm biopsy punch and a no. 10 scalpel blade. The donor animals were Labrador Retrievers from the colony housed at the Baker Institute for Animal Health and the shoulders were macroscopically normal at the time of necropsy. The explants were washed three times with Gey's balanced salt solution (Sigma Chemicals, St. Louis, MO) and transferred to serum-free Ham's F12 medium (Gibco, Carlsbad, CA) for culture. Medium was supplemented (per 100 ml) with 2.5 ml HEPES (1 M), 1.0 ml alpha keto glutarate (3 mg/ml), 1.0 ml calcium chloride (4.85 g/ml), 200 μl gentamycin (10 mg/ml), 200 μl penicillin/streptomycin (10 000 U/ml Pen-G, 10 000 μg/ml streptomycin sulphate) and 400 μl Fungizone (250 μg/ml). After filtering through a 0.22 μm filter, this was supplemented with ITSCR+ premix (Collaborative Biosciences, Bedford, MA) and immediately before changing the media, it was supplemented with 1.0 ml L-glutamine (30 mg/ml) and 1.0 ml ascorbic acid (50 mg/ml). Explants were cultured for 48 h prior to loading at 37°C, 79% humidity and 5% CO_2.

CYCLIC IMPACT LOADING

Cyclic loads were applied to the central 2 mm of the 4 mm cartilage explants by means of our mechanical loading machine, which has been described previously[16,17]. The loading machine allows pneumatically controlled testing of samples in triplicate whilst housed in an incubator. Labview 6 programming software (National Instruments, Austin, TX) enables load control and data acquisition through a computer equipped with a PCI-M10-16E4 Data Acquisition Board. Explants were loaded in stainless steel chambers and were held in place by stainless steel rings. The indenters were non-porous and also of stainless steel. Loading chambers were filled with Gey's balanced salt solution for the duration of the loading. Loading was for 120 min at a magnitude of 5 MPa. The frequency of loading was 0.3 Hz, of which active loading lasted for 1.0 s per cycle. Loading in this square waveform produced a stress rate of 60 MPa/s. Following loading, explants were washed three times with Gey's balanced salt solution and transferred to the serum-free Ham's F12 culture medium. Culture medium was changed every 2 days and was stored at −20°C until analysis.

INDUCIBLE NO SYNTHASE INHIBITION: EFFICACY OF L-NAME INHIBITOR IN CARTILAGE

In order to determine whether NO plays a role in the spread of cell death, we first tested the efficacy of the iNOS inhibitor, L-N^G-nitroarginine methyl ester HCl, L-NAME (Biomol Research Labs, Plymouth Meeting, PA). To test that the L-NAME could diffuse into the cartilage effectively, six cartilage discs were halved, one half was incubated in 25 μg/ml lipopolysaccharide (LPS) and one half in LPS plus the addition of either 1 mM or 10 mM L-NAME. Three cartilage discs were also cultured in medium alone. LPS is known to induce glycosaminoglycan (GAG) release from cartilage in a pathway mediated by NO[18]. Discs were cultured for 48 h and then media were collected for GAG analysis using the DMMB assay (as previously described)[19].

INDUCIBLE NO SYNTHASE INHIBITION IN LOADED CARTILAGE

To test whether the inhibition of iNOS prevents the spread of cell death in loaded cartilage, nine cartilage discs were loaded as described above. That is, each 4 mm disc was placed within the chamber of the loading machine and the centre was loaded with a 2 mm diameter indenter. Immediately post-load, the discs were halved down the centre of the 2 mm loaded core region. One half of the disc was cultured in medium alone and the other half was cultured in medium supplemented with 10 mM L-NAME. Three discs were cultured for 7 days and six for 21 days with media changed every 2 days. Alternatively, discs were loaded in the presence of L-NAME and the L-NAME was removed from one of the halves immediately post-load. Nine discs served as unloaded controls, which were halved, as the loaded samples, and cultured in medium alone or supplemented with 10 mM L-NAME inhibitor. Media samples were stored at −20°C until analysis for GAG content (DMMB assay) or total nitrite concentration (Griess reaction). Cartilage samples were embedded in OCT mounting medium, frozen in liquid nitrogen, and stored at −80°C until analysis of cell death using the TUNEL assay.

CASPASE INHIBITION: EFFICACY OF Z-VAD FMK INHIBITOR IN CARTILAGE

In order to determine whether the spread of cell death post-load is due to apoptosis or necrosis, we first tested the ability of the broad-spectrum caspase inhibitor, Z-VAD FMK (R & D Systems, Minneapolis, MN) to inhibit cell death in response to a known apoptotic stimuli. To establish the ability of the inhibitor to diffuse into cartilage we halved three 4 mm cartilage discs. One half was cultured in medium supplemented with 50 μg/ml mitomycin-C (MMC; from Streptomyces Caespitosus, Sigma Chemicals, St. Louis, MO) which is known to induce apoptosis. The other half of the cartilage disc was cultured in 50 μg/ml MMC plus 100 μM Z-VAD FMK. Three cartilage discs were cultured in medium alone to serve as controls. Following 48 h in culture, cartilage was embedded in OCT medium, frozen in liquid nitrogen, and stored at −80°C for TUNEL assay analysis.

CASPASE INHIBITION BY Z-VAD FMK IN LOADED CARTILAGE

To test whether caspase inhibition prevents the spread of cell death in loaded cartilage explants, nine cartilage discs were loaded as described above. Immediately post-load the discs were halved down the central 2 mm loaded core region. One half of the disc was cultured in medium alone and the other half was cultured in medium supplemented with 100 μM Z-VAD FMK. Three discs were cultured for 7 days and six for 21 days with media changed every 2 days. Nine discs served as unloaded controls, which were halved as the loaded samples, and cultured in medium alone or

supplemented with 100 μM Z-VAD FMK inhibitor. Media samples were stored at −20°C until analysis for GAG content (DMMB assay). Cartilage samples were embedded in OCT mounting medium, frozen in liquid nitrogen, and stored at −80°C until analysis of cell death using the TUNEL assay.

TUNEL LABELLING

Frozen cartilage samples, embedded in OCT medium, were cut into 6 μm thick cross-sections using a Microm HM505E cryostat, at −27°C. Sections were collected onto slides coated with chrome alum gelatin. Three sections were adhered to each slide and three slides were analysed per explant. The three slides were taken from different depths in the explants. Cell death was quantified using the Fluorescein Labelled *In Situ* Cell Death Detection Kit (Roche Diagnostics). The kit was performed according to manufacturer's instructions with a few modifications. Specifically, cartilage was fixed for 30 min in 4% paraformaldehyde in phosphate buffered saline (PBS). Samples were then washed in fresh PBS for a further 30 min. Sections were permeabilised for 2 min with 0.1% Triton X-100 in 0.1% sodium citrate. After washing in PBS, samples were digested for 2 min with 20 μg/ml Proteinase-K solution. Labelling was with a 1:100 dilution of the terminal deoxynucleotidyl transferase (TdT) enzyme in the manufacturer's diluent. Incubation was for 1 h at 37°C. Positive controls were added by digesting cartilage sections for 20 min, at 37°C, with 1 mg/ml Dnase I (Sigma). Sections were mounted using Vectorshield mounting medium for fluorescence containing propidium iodide (Vector Labs, Burlingame, CA). This enables total cell count to be determined. Cells were viewed with an Olympus IX70 confocal laser scanning fluorescent microscope, attached to a PC running Fluoview 2.1 software, with appropriate filters for fluorescein and propidium iodide. Images were captured under 10× magnification as both isolated fluorescein images and merged images. This allowed for counting of TUNEL positive cells and total cell counts. Scion Image version 1.62 software was used to count cells.

TOTAL NITRITE MEASUREMENT

NO release can be determined by measuring the accumulation of its stable degradation products, nitrite and nitrate, in the culture media. First, nitrate was reduced to nitrite, which was then quantified using the Griess Reagent Kit (Molecular Probes, Eugene, OR). For nitrate reduction, 150 μl aliquots of media were incubated for 15 min at 37°C in the presence of 0.1 U/ml nitrate reductase (Sigma), 50 μM NADPH and 5 μM FAD in a final volume of 160 μl. To avoid interference of the NADPH with the assay, NADPH was subsequently oxidised. For this purpose, samples were incubated with 10 U/ml lactate dehydrogenase and 10 mM sodium pyruvate for 5 min at 37°C in a final volume of 170 μl. The Griess Reaction was then carried out according to the manufacturer's instructions.

STATISTICAL METHODS

Differences in cell death between treated and untreated explants were performed using paired two-tailed *t* tests. Differences in GAG and NO release were analysed using the General Linear Model (Minitab Statistical Software, Minitab Inc.) with respect to time of culture post-loading and presence or absence of inhibitors. A *P* value less than 0.05 was considered significant.

Results

THE ROLE OF NO IN THE SPREAD OF CELL DEATH IN IMPACT DAMAGED CARTILAGE: EFFICACY OF L-NAME INHIBITOR IN CARTILAGE

Preliminary experiments determined that L-NAME is capable of diffusing into cartilage and working efficiently. Addition of 25 μg/ml LPS to culture medium caused a significant increase in GAG loss from the cartilage over 48 h. Addition of L-NAME reduced GAG loss in a dose-dependent manner. The addition of 10 mM L-NAME reduced GAG loss to levels similar to that of controls (Fig. 1). For this reason, all further experiments were carried out using 10 mM L-NAME supplemented media.

GAG RELEASE INTO CULTURE MEDIUM

GAG release into the culture medium was quantified using the DMMB assay. GAG release decreased over the 21 day culture period. GAG release from loaded cartilage was greater than from control cartilage ($P < 0.05$) with most of the difference obvious in the first week after loading (Fig. 2). L-NAME induced decrease in GAG release was apparent during the third week of culture ($P < 0.05$).

NITRITE RELEASE INTO CULTURE MEDIUM

Total nitrite released into the culture medium during the 21 day post-load incubation was measured at regular intervals. Fig. 3 shows that there was no significant difference in the nitrite released between the loaded and control cartilage ($P = 0.57$). There was a significant ($P < 0.05$) reduction in nitrite release to the culture medium when

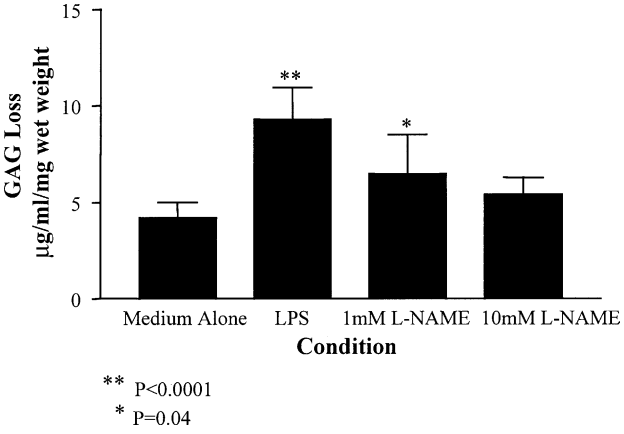


Fig. 1. Effect of LPS and L-NAME on GAG loss from articular cartilage explants. Cartilage was treated for 48 h with 25 μg/ml LPS in the presence or absence of L-NAME, as described in Methods. GAG present in the culture medium was quantified with the DMMB assay. LPS significantly ($P < 0.0001$) increased GAG loss when compared to media only control. The addition of 1 mM L-NAME reduced the amount of GAG loss, but it was still significantly ($P = 0.04$) elevated over control. When cultured in LPS plus 10 mM L-NAME no significant increase in GAG loss over control was observed. Thus the iNOS inhibitor, L-NAME, can diffuse into cartilage and reduce lipopolysaccharide (LPS) induced GAG loss from cartilage.

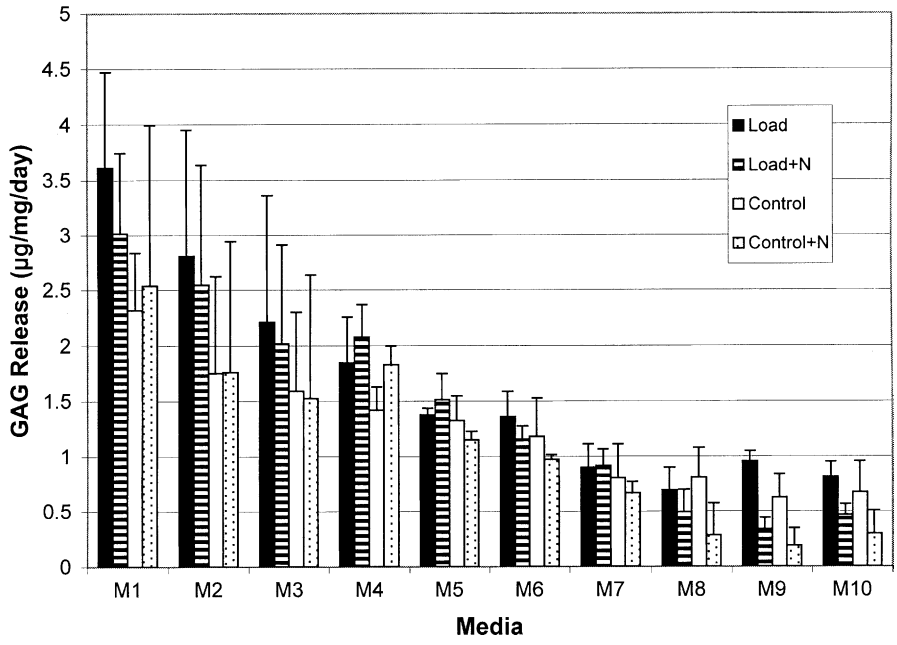


Fig. 2. Effect of impact load and L-NAME on GAG loss from articular cartilage. Four millimetre cartilage discs were harvested and loaded as described in Methods. After loading, loaded discs and never loaded controls were cultured for 21 days in the presence or absence of L-NAME. Conditioned media was replaced with fresh media every 2 days and frozen at −20°C until analysed for GAG content by DMMB assay. There was a decrease in GAG release over the 21 day culture period. L-NAME decreased GAG release only during the third week of culture ($P < 0.05$). Each bar represents a mean value for $n = 6$ cartilage samples at each media collection (every 2 days). +N indicates that 10 mM L-NAME was added to the culture media. M1 represents the first media collection, at which time the explants were fed. M2 represents the second media collection 2 days later, etc.

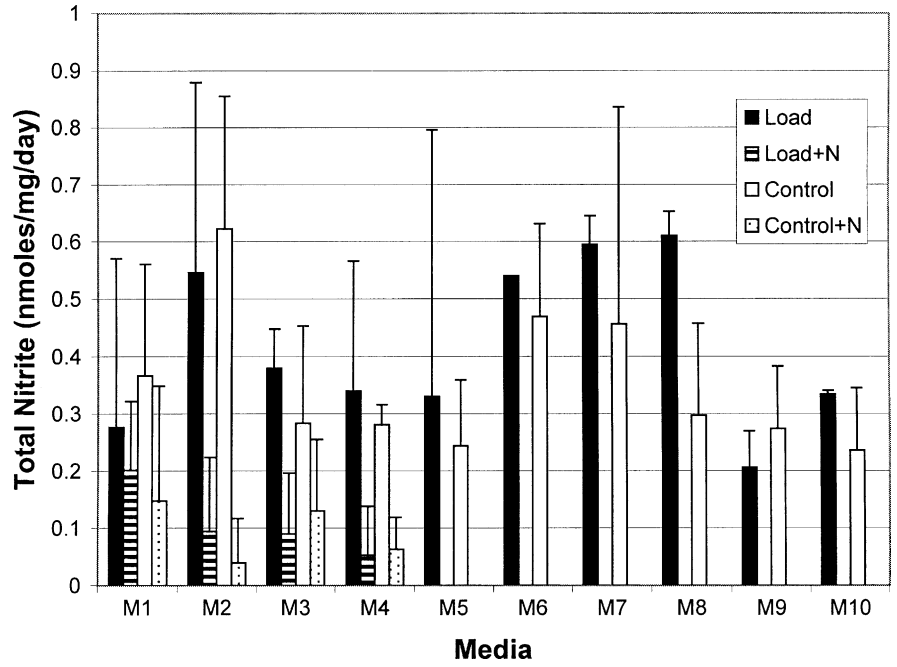


Fig. 3. Effect of impact load and L-NAME on nitrite released into the culture media. Cartilage discs were treated as for Fig. 2. Nitrites in the culture media were measured using the Griess reaction. There was no difference in nitrite release between loaded and control cartilage. Addition of 10 mM L-NAME to the culture media significantly reduced the nitrite released by both loaded and control explants. Each bar represents a mean value for $n = 6$ cartilage samples at each media collection (every 2 days). M1 represents the first media collection, at which time the explants were fed. M2 represents the second media collection 2 days later, etc.

control or loaded explants were cultured in the presence of 10 mM L-NAME. Nitrite release in the presence of L-NAME became undetectable by the second week of culture. This further documents the efficacy of L-NAME in both the control and loaded cultures and the insensitivity of nitrite levels to load.

INHIBITION OF INDUCIBLE NO SYNTHASE IN LOADED CARTILAGE FAILS TO PREVENT THE SPREAD OF CELL DEATH

After 7 days post-load, cell death was significantly increased in the core of loaded cartilage, as expected, and this was not prevented by the addition of 10 mM L-NAME. Control cartilage incubated in 10 mM L-NAME showed a significantly increased level of cell death when compared with medium only control [$P = 0.015$, paired two-tailed t test, Fig. 4(a)]. After 21 days post-load, cell death was significantly increased in the ring as well as the core cartilage if core and ring remained contiguous, as previously reported[9]. The presence of 10 mM L-NAME throughout the 21 days of culture did not prevent cell death in either the core or the ring [Fig. 4(b)]. This was true whether the L-NAME was present during loading or added immediately after. Values of cell death in control, loaded core and surrounding ring cartilage after 7 and 21 days are shown in Table I. Inability of the iNOS inhibitor, L-NAME, to prevent cell death following load suggests that NO is not mediating the spread of cell death in the impact damaged cartilage.

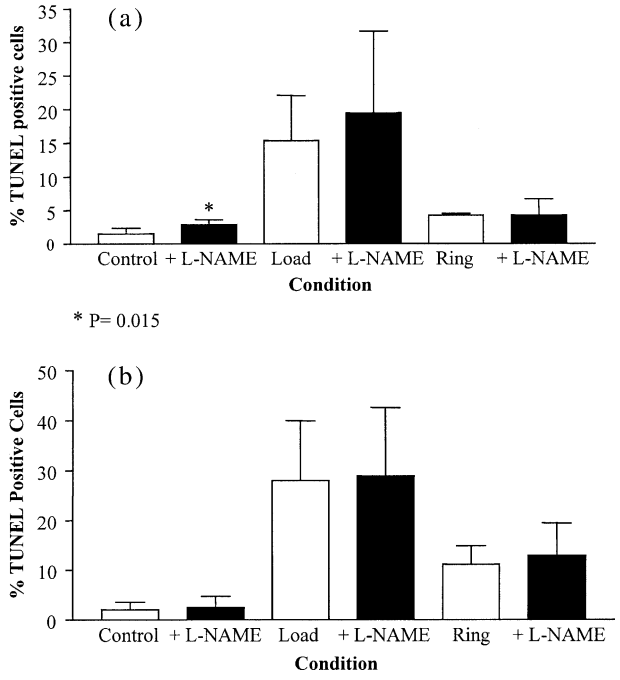


Fig. 4. Effect of L-NAME on cell death in cartilage. Four millimetre cartilage discs were loaded as described in Methods. Nine discs were halved across the 2 mm core and one half was cultured in media alone, the other half was cultured in the presence of 10 mM L-NAME. Nine discs served as never loaded controls. They were halved and cultured as above. Three sets of discs (control and loaded, plus or minus L-NAME) were cultured for 7 days. Six sets were cultured for a total of 21 days. (a) Seven days post-load. After 7 days post-load, cell death was significantly increased in the core of loaded cartilage. This was not prevented by the addition of 10 mM L-NAME. Control cartilage incubated in 10 mM L-NAME showed a significantly increased level of cell death when compared with medium only control ($P = 0.015$, paired two-tailed t test). (b) Twenty-one days post-load. After 21 days post-load, cell death was significantly increased in the surrounding ring as well as the loaded core cartilage. This was not prevented by culture in 10 mM L-NAME. The presence of L-NAME did not alter levels of cell death in the control explants.

THE ROLE OF CASPASE MEDIATED APOPTOSIS IN THE SPREAD OF CELL DEATH IN IMPACT DAMAGED CARTILAGE: EFFICACY OF Z-VAD FMK INHIBITOR IN CARTILAGE

To ascertain whether Z-VAD FMK can enter cartilage and be effective, cartilage was cultured in the presence of 50 μg/ml MMC in the presence or absence of 100 μM Z-VAD FMK for 48 h. The MMC caused a significant increase in cell death when compared with cartilage cultured in medium alone. This increase was prevented by the addition of Z-VAD FMK to the medium (Fig. 5).

THE BROAD-SPECTRUM CASPASE INHIBITOR, Z-VAD FMK, FAILS TO PREVENT CELL DEATH IN IMPACT DAMAGED CARTILAGE

After 7 days post-load cell death was increased in the loaded core. The presence of 100 μM Z-VAD FMK did not alter levels of cell death in loaded core, in the surrounding ring, or in the control explants [Fig. 6(a)]. After 21 days post-load cell death was increased in both loaded core and surrounding ring. The presence of Z-VAD FMK did not change levels of cell death in either the core or surrounding ring [Fig. 6(b)]. Values of cell death in control, loaded and unloaded ring cartilage after 7 and 21 days are shown in Table II. These data suggest that the cell death we are observing in our system is not caspase mediated.

GAG RELEASE INTO CULTURE MEDIUM

GAG release into the culture medium was quantified using the DMMB assay. GAG release decreased over the 21 day culture period but was not significantly different between loaded and control cartilage (data not shown). Z-VAD FMK had no significant effect on the GAG release from loaded or control cartilage.

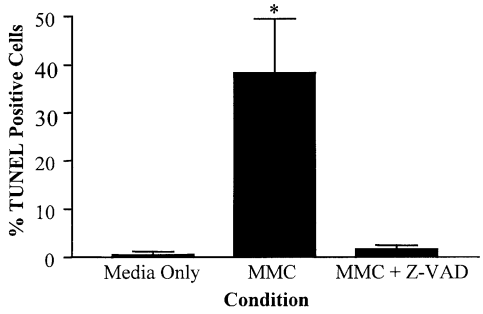
Discussion

Increased levels of both NO and apoptosis have been linked with the OA disease process in humans. The evidence for the increased NO levels seen in OA seems

Table I

Effect of L-NAME on cell death in cartilage. Mean percent cell death (standard deviation) in control, loaded core and unloaded ring cartilage 7 and 21 days post-load. Cartilage was incubated in medium alone or medium supplemented with 10 mM L-NAME

Cartilage	Medium alone	+10 mM L-NAME
7 days post-load ($n = 3$)		
Control	1.5 (0.9)	2.8 (0.8)
Loaded core	15.3 (6.7)	19.4 (12.2)
Unloaded ring	4.3 (0.3)	4.3 (2.4)
21 days post-load ($n = 6$)		
Control	2.0 (1.6)	2.5 (2.3)
Loaded core	28.0 (12.0)	28.9 (13.7)
Unloaded ring	11.2 (3.7)	12.9 (6.6)



* P=0.0009 (unpaired 2-tailed t-test, compared with medium only control).

Fig. 5. Effect of Z-VAD FMK on mitomycin-C (MMC) treated cartilage. Three 4 mm cartilage discs were halved. One half was cultured in the presence of MMC, a known apoptotic stimulus. The other half was cultured in MMC plus Z-VAD FMK. Three discs served as controls. The addition of MMC to the media caused a significant ($P = 0.0009$) increase in cell death in cartilage explants when compared with medium only controls. This increase could be prevented by the addition of Z-VAD FMK to the medium.

clear, with elevated levels found in serum, synovial fluid and cartilage[20,21] as well as the synovial membrane[22]. However, the role of NO in cell death seems more complex. NO has been shown to induce chondrocyte apoptosis *in vitro*[13] and apoptotic chondrocytes have been shown to stain positive for the iNOS enzyme in joints affected with rheumatoid arthritis. The iNOS inhibitor, L-NMMA, reversed the apoptotic pathway[12]. In contrast, we have shown that the iNOS inhibitor L-NAME had no influence on the cell death in cyclically loaded explants. This is in keeping with the observation that in OA cartilage the prevalence of NO in apoptotic cells was no different to that in non-apoptotic cells[23]. This suggests that NO is not the initiating signal for apoptosis *in vivo*. Also in agreement, when chondrocytes were transduced with the iNOS gene, the endogenously produced NO inhibited matrix synthesis, as expected, however, there was no evidence of cell death[24].

In animal models of arthritis the data is also contradictory. In a canine anterior cruciate ligament transection model of OA, the addition of NOS inhibitors led to a reduction in the levels of chondrocyte apoptosis[25]. Mice deficient in iNOS have also been shown to be protected against an inflammatory arthritis model[26]. However, in a murine instability model of OA, iNOS knockout mice were shown to be more susceptible to OA[27]. Also suggesting no link between apoptosis and NO, in a rabbit transection model of OA it was found that intra-articular injections of hyaluronan decreased levels of apoptosis but not NO[28].

The apparent contradictory nature of these findings may be explained by an interesting relationship between apoptosis, necrosis and NO described by Blanco *et al.*[13] who point out that oxygen radicals, which themselves induce necrosis, react with NO. In this situation, the oxygen radicals can reduce NO-induced apoptosis. In turn, NO protects against oxygen radical induced necrosis. In such a situation, oxygen radical scavengers will increase apoptosis while inhibitors of NO production will favour necrosis. Del Carlo and Loeser[29] report that NO mediated cell death requires generation of reactive oxygen species, with NO itself not being toxic to cells. Compounds that only release NO, even at high concentrations, did not cause cell death. Depending on the redox status of the cell, NO was even found to be protective.

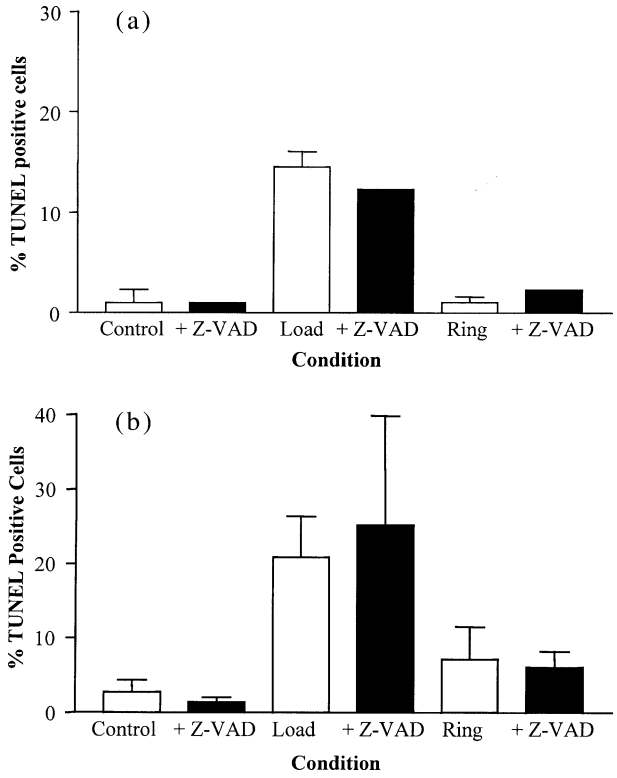


Fig. 6. Effect of Z-VAD FMK on cell death in cartilage. Four millimetre cartilage discs were loaded as described in Methods. Nine discs were halved across the 2 mm core and one half was cultured in media alone, the other half was cultured in the presence of 100 μM Z-VAD FMK. Nine discs served as never loaded controls. They were halved and cultured as above. Three sets of discs (control and loaded, plus or minus Z-VAD FMK) were cultured for 7 days. Six sets were cultured for a total of 21 days. (a) Seven days post-load. After 7 days post-load cell death was increased in the loaded core. The presence of 100 μM Z-VAD FMK did not alter levels of cell death in loaded core, in the surrounding ring, or in the control explants. (b) Twenty-one days post-load. Six cartilage discs were cultured in the presence or absence of Z-VAD FMK, as described in (a). After 21 days post-load, cell death was significantly increased in the surrounding ring as well as the loaded core cartilage. This was not prevented by culture in Z-VAD FMK. The presence of 100 μM Z-VAD FMK did not alter levels of cell death in the control explants.

One of the suggested mechanisms leading to the increased production of NO in OA is mechanical stress. Fermor *et al.*[30] show increased NO and iNOS production in porcine articular cartilage explants subjected to both static and intermittent compression. There was no loss of cell viability seen. Similarly, Das *et al.*[31] showed that shear stress increased NO levels in cultured bovine chondrocytes. Lee *et al.*[32] also showed increased NO production in OA chondrocytes embedded in agarose subjected to shear stress but decreased NO production by intermittent hydrostatic compression. In agreement, Wiseman *et al.*[33] found that dynamic compression of equine chondrocytes in agarose constructs inhibited NO production. In this current study, we have shown that cyclic impact loading of cartilage explants had no effect on nitrite released to culture media (Fig. 3). This suggests that the loading regimen, as well as the environment of the chondrocytes, is important in determining the chondrocyte response. Some of the apparent difference may also be explained by the

Table II
Effect of Z-VAD FMK on cell death in cartilage. Mean percent cell death (standard deviation) in control, loaded core and unloaded ring cartilage 7 and 21 days post-load. Cartilage was incubated in either medium alone or medium supplemented with 100 μM Z-VAD FMK

Cartilage	Medium alone	+100 μM Z-VAD FMK
7 days post-load ($n = 3$)		
Control	1.0 (1.3)	1.0 (0.0)
Loaded core	14.6 (1.5)	12.3 (0.0)
Unloaded ring	1.1 (0.6)	2.3 (0.0)
21 days post-load ($n = 6$)		
Control	2.7 (1.6)	1.4 (0.6)
Loaded core	20.9 (5.4)	25.2 (14.6)
Unloaded ring	7.2 (4.4)	6.1 (2.1)

magnitude of loading employed. In contrast to Fermor *et al.*[30] who used only 0.5 MPa, we used loads of 5 MPa. A load of 5 MPa is within the physiological range experienced by the cartilage. Studies in humans suggest that the compressive stresses acting on articular cartilage lie in the range of 5–8 MPa during level walking[34] and are as high as 18 MPa when rising from a chair[35]. Studies in the pig suggested that the contact stresses in quadrupeds was 0–2 MPa lower than in humans[36].

GAG release during the first 2 weeks did not reflect an NO-induced degradation although this changed during the third week. In previous studies from our laboratory we observed a net loss of GAG content 1 day after impact but the maximum load was 10 MPa. GAG release to medium was not looked at. Sah *et al.*[37] reported loss of proteoglycans to the medium which was greatest soon after loading. They used slow, displacement controlled cyclic compression with a peak stress ~1 MPa. In a recent experiment, Thibault *et al.*[38] reported statistically significant increase in GAG in the medium in the first 4 days after loading with no difference thereafter. No net loss of PG was found. This loading protocol again differed somewhat. It was unconfined compression over the whole disc with cartilage on bone. Stress reached similar levels (5–8 MPa) and rate of loading was rapid. In spite of some differences in protocol, the results shown in Fig. 2 are in general agreement with the observation that GAG release to the medium is increased in response to load. However, in additional experiments, differences in GAG release from control and loaded specimens did not reach significance. Proteoglycan synthesis in cartilage has been shown to be increased in response to dynamic load[16]. Previous studies[17,39] suggest that proteoglycan synthesis is increased in live cells to compensate for the loss of synthesis due to cell death in loaded cartilage. Previous work in this loading system showed that the response of proteoglycan synthesis depended on the duration of loading. Explants loaded for 2 min showed a 76% increase in sulphate incorporation after 2 days in culture and 104% increase after 10 days. In contrast 2 h of loading, as used in the present study, decreased sulphate incorporation by 41% after 10 days culture[17]. However, this decrease could reflect the fact that the number of viable cells has decreased. In this study we have no measure of the total GAG content or newly synthesised GAG in cartilage and cannot conclude anything about proteoglycan synthesis.

The second hypothesis of this paper was that the spread of cell death from loaded cartilage was apoptotic. However, the spread of cell death after 21 days could not be prevented using the broad-spectrum caspase inhibitor, Z-VAD FMK. This suggests that the spread of cell death is not caspase mediated apoptosis. This is in contrast to D'Lima *et al.*[40] who found a significant increase in the number of apoptotic cells 96 h post-load, which could be inhibited by Z-VAD FMK.

In these experiments, we used the TUNEL assay to detect cell death. This assay was previously thought to be specific for apoptotic cells, but it is now thought that it will label necrotic cells positive too[41]. Previously, in impact damaged cartilage both necrosis and apoptosis have been documented. Necrosis appeared early while apoptosis appeared to be a later response[6]. It is relevant that in other systems, the same signal may trigger either apoptosis or necrosis depending on the intensity. Low levels of peroxynitrite, formed from the reaction between NO and superoxide anion (O_2^-), triggered apoptosis in cultures of cortical neurones while higher levels led to necrosis[42]. A form of death distinct from apoptosis or necrosis has been reported in neuronal cells. Cell shrinkage and plasma membrane blebbing occurred although nuclei remained intact and there was no DNA fragmentation[43]. Sperandio *et al.*[44] described a form of non-apoptotic cell death and dubbed it 'paraptosis'. It is programmed death, requiring the need for gene expression and *de novo* protein synthesis, and yet lacks responsiveness to Z-VAD FMK. It fails to show the characteristic nuclear fragmentation of apoptosis, and is more like necrosis with cytoplasmic vacuolation and mitochondrial swelling. Sperandio *et al.*[44] claim that these parapoptotic cells are TUNEL negative. Although our cells are TUNEL positive, the caspase-independent cell death we observe may still be paraptosis. It may be the case that we are detecting late stage paraptosis when the cells are undergoing secondary necrosis.

In conclusion, this paper indicates that the spread of cell death from loaded cartilage to unloaded cartilage is not classic apoptosis and is not mediated by NO. There are other soluble signalling molecules which may be responsible for the spread of cell death. These include catabolic cytokines such as IL-1β and TNFα or FAS/FAS ligand. TNFα is especially interesting since it can induce apoptosis in chondrocytes[45] and has been found in the synovial membrane[46] as well as the cartilage of OA patients[47]. Additionally, type I TNF receptors have been shown to be modulated by mechanical loading[48].

Furthermore, cell death initiated by signalling through death domain receptors, as is the case for FAS ligand and TNFα, can result either in apoptotic or necrotic cell death, depending on the availability of active caspases. Further studies into the nature of the intercellular signal in our *in vitro* loaded samples have important implications for the spread of cartilage degeneration in OA *in vivo*. They could lead to the identification of important therapeutic targets to slow the progression of the disease.

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